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Formation of Competent *Bacillus subtilis* Cells†

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The process of competent cell formation for transformation has been studied with early-stationary-phase (T_1) cells of *Bacillus subtilis* which had been grown in an enriched Spizizen minimal medium and transferred to a second synthetic medium. Rifampin, chloramphenicol, and tunicamycin were strong inhibitors of competent cell formation, as well as vegetative growth. After formation, competent cells were no longer sensitive to the above agents. Methicillin and an inhibitor of chromosomal replication, hydroxyphenylazouracil, did not inhibit the development of competence. A D-alanine-requiring mutant strain developed competence even in the absence of D-alanine in the second medium. A T_1-stage culture showed the activity of extracellular serine protease which is necessary for sporulation. Competent cell formation was completely blocked by 0.7 M ethanol, which is a specific inhibitor of early events during sporulation, including forespore septum formation. Competent cells were formed even in media which supported sporulation. The development of competence was also studied with *spo0* mutants at 10 different loci. Most *spo0* mutations repressed the development of competence except for *spo0C*, *spo0G*, and *spo0J*. These results suggest that competent cells are formed from early sporulating cells with the synthesis of cell wall materials and by factors whose genes are activated by the supply of nutrients. It is suggested that common steps are involved both in forespore septation and in competent cell formation.

Transformation in *Bacillus subtilis* is mediated by competent cells which are metabolically less active and smaller in cell size and have a lower buoyant density (for a review, see references 20 and 33). Competent cells develop during early stationary phase (6) or in a fresh medium to which early-stationary-phase cells are transferred (2), depending on the composition of the medium. The supernatant of competent cultures seems to contain factors which induce competence in noncompetent cultures, and these are assumed to be autolysins. Although there is a theory (1) of mesosomal uptake of transforming DNA and there are some suggestions (29, 31) that the development of competent cells is related to the sporulation process because some asporogenous mutants showed poor competence, questions still remain as to how the competent cells are formed and how the competence-inducing factors function (20, 33).

In the present communication, we examine the conditions necessary for the formation of competent cells from early-stationary-phase cells and discuss the possibility that competent cells are formed during cell division in an early stage of sporulation.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are described in Table 1. Strain NIG1121M (*met*) was used as a recipient strain in most experiments. Strain 168T (*thyA1 thyB1*) (25) was used as a donor for transformation.

Transformation procedures. The competent cells were prepared by the methods described by Dubnau et al. (10), which are essentially the same as those described originally by Anagnostopoulos and Spizizen (2). Incubation was at 37°C in all experiments. An overnight culture of the recipient strain grown in Penassay broth (Difco Laboratories, Detroit, Mich.) was added as a 5% (vol/vol) inoculum into SPI medium, which contained Spizizen minimal salts-glucose supplemented with 0.02% Casamino Acids (Difco), 0.1% yeast extract (Difco) and 50 μg of amino acid per ml. The cells were grown to the end of log phase and then diluted 10-fold into fresh SPII medium, which was made by adding 0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ to SPI medium. The growth of cells was followed as described before (26). A maximal competence was obtained around 90 min after the dilution of the first culture. A competent culture of 1 ml was treated with 5.8 μg of transforming DNA per ml for 30 min followed by DNase (50 μg/ml) treatment for 10 min. The number of viable cells was scored on a broth-supplemented Vogel-Bonner minimal salts-glucose medium (25) solidified with 1.5% Difco agar. Difco nutrient broth was added at a concentration of 1% (vol/vol). Amino acid was supplied at a concentration of 20 μg/ml. The

TABLE 1. Bacterial strains

Strain[a]	Genotype	Source of *spo0*
168T	*thyA1 thyB1*	
QB928	*arol906 purB33 dal-1 trpC2*	
NIG1121M	*met*	
NIG1121	*met his*	
NIG1131	*met his spo0A34*	Piggot
NIG1132	*met his spo0B136*	Piggot
NIG1133	*met his spo0C9*	Piggot
NIG1134	*met his spo0D8*	Piggot
NIG1135	*met his spo0G14*	Piggot
NIG1136	*met his spo0H17*	Piggot
NIG1137	*met his spo0J87*	Piggot
NIG1138	*met his spo0K141*	Piggot
NIG1139	*met his spo0E81*	Kawamura
NIG1140	*met his spo0F221*	Kawamura

[a] Strain QB928 (9) was obtained from H. Saito through Y. Fujita. Strain NIG1121M is a His^+ transformant of NIG1121 (26). All *spo0* strains are Leu^+ Spo^- transformants of strain HA101 (*met his leu*) (22) with DNA from original *spo0* mutants. Original *spo0* strains (23) are SL566 (*spo0A34 phe-12 rif-2 tal-1*), SL964 (*spo0B136 metC3 tal-1*), SL966 (*spo0C9 metC3 tal-1*), SL225 (*spo0D8 metC3 tal-1*), SL221 (*spo0G14 metC3 tal-1*), SL513 (*spo0H17 rif-2 trpC2*), SL670 (*spo0J87 metC3 tal-1*), SL741 (*spo0K141 trpC2*), SCR162 (*spo0E81 trpC2 lys-3*), and UOT274 (*spo0F221 metB5 nonB1*).

Vogel-Bonner minimal salts medium was used as a dilution buffer.

The competent cells were also prepared in the synthetic medium of Schaeffer et al. (30). Schaeffer's basal medium contained 1.05% K_2HPO_4, 0.35% KH_2PO_4, 0.005% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, and 0.0005% $MnCl_2 \cdot 4H_2O$. The basal medium was supplemented with appropriate carbon and nitrogen sources as described in the text.

Isolation of transforming DNA. DNA was purified by the phenol method (28) from cells of strain 168T grown as described elsewhere (25), and its concentration was determined by its absorbance at 260 nm.

Chemicals. Rifampin, chloramphenicol, and methicillin were purchased from Boehringer Mannheim Corp., New York, N.Y., Sankyo, Tokyo, Japan, and Banyu Co. Ltd., Tokyo, Japan, respectively. Tunicamycin and hydroxyphenylazouracil were kindly supplied by G. Tamura and N. Cozzarelli, respectively. 1,10-Phenanthroline and phenylmethylsulfonyl fluoride were purchased from Wako, Tokyo, Japan, and Sigma Chemical Co., St. Louis, Mo.

RESULTS

Development of competence in a synthetic medium devised for transformation. The competence of a culture expressed as the number of Met^+ transformants per recipient cell developed during vegetative growth in SPI medium and declined toward the end of log phase (Fig. 1). The dilution of the culture at the end of log phase with fresh SPII medium conferred an elevated competence to the culture about 90 min after dilution. The dilution of the culture later than 2 h after the end of log phase was less effective for developing competence (Table 2).

Therefore, with the medium employed in the present experiments, the development of competence was biphasic. Competent cells appeared during log phase as well as after the transfer of late-log-phase cells to SPII medium. The number of viable cells did not increase immediately after dilution in contrast to an immediate increase in turbidity. The eventual number of transformants increased about 100 times in SPII medium. Since competent cells are penicillin resistant (19) and nondividing, the increase in transformants must mean that new competent cells were being formed in SPII medium.

In the experiment described above, a compe-

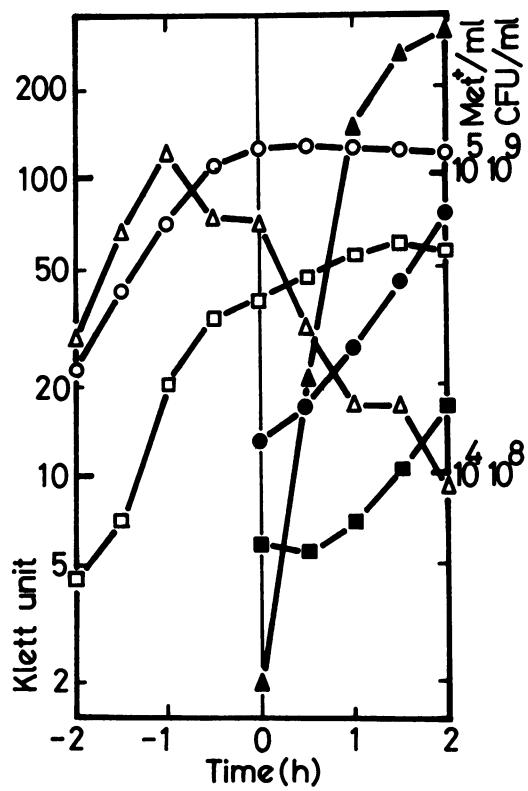

FIG. 1. Development of competence in SPI and SPII medium. An overnight culture of NIG1121M in Penassay broth was inoculated in fresh SPI medium 5% (vol/vol). At the end of the log phase, a portion of the culture was diluted 10-fold into fresh SPII medium. One milliliter of the culture was taken and incubated with DNA to examine competence. Symbols: ○, turbidity in SPI medium; ●, turbidity in SPII medium; △, Met^+ transformants in SPI medium; ▲, Met^+, transformants in SPII medium; □, CFUs in SPI medium; ■, CFUs in SPII medium.

TABLE 2. Dependence of competence development on the length of incubation of the cultures in SPI medium

Stage[a]	No. of Met$^+$ transformants per ml (10^3)	CFU per ml (10^7)	Frequency of transformation (10^{-4})
T_0	260	32.0	8.1
$T_{0.5}$	93.0	21.6	4.3
T_1	71.0	15.9	4.5
$T_{1.5}$	70.0	12.9	5.4
T_2	20.0	13.1	1.5
$T_{2.5}$	5.00	10.3	0.49

[a] The symbol T_n means n hours after the end of the log phase. At the indicated time, NIG1121M cells grown in SPI medium were diluted 10-fold with fresh SPII medium. Competence was measured after 90 min by adding DNA to the culture.

tent culture was incubated with DNA for 30 min. During such a long period, new competent cells may be formed or competent cells already formed may lose competence, giving an erroneous estimation of the number of competent cells at a specific time. To examine the rate of increase of competent cells, DNA was added to a competent culture for 3 min, followed by DNase treatment for 2 min. The number of competent cells existing at the time of dilution decreased with further incubation (Fig. 2). Eventually the number of transformants began to increase about 45 min after dilution, roughly in accordance with the onset of viable cell division.

Blocking of competent cell formation by inhibitors of RNA and protein or cell wall synthesis. We next examined conditions for the development of competence in SPII medium. To reduce the initial number of competent cells already formed at the time of transfer, the culture was diluted 1 h after the end of log phase when the competence of the first culture had begun to decline.

The inhibitors of DNA, RNA, and protein or cell wall synthesis were used at concentrations as low as possible. These drugs were added at the time of dilution, and the increase of competent cells was examined 60 min after dilution by adding DNA to the culture. In control experiments, the drugs were added with the transforming DNA 60 min after dilution, and the relative frequency of transformation was compared under these two conditions (Table 3).

Hydroxylphenylazouracil (HPUra) (12, 17), which is a specific inhibitor of DNA polymerase III of *B. subtilis*, was not effective at a concentration of 10 μM (and even at 100 μM; data not shown). New competent cells were formed in the presence of the inhibitors, whereas an increase in the number of the majority of noncompetent cells was blocked. Therefore, the formation of competent cells does not require chromosome replication, which is necessary for cell multiplication. This conclusion is supported by the finding that the formation of competent cells was seen in thymine-requiring strains in the absence of thymine (data not shown: 3).

Rifampin (0.01 μg/ml) and chloramphenicol (1.0 μg/ml), however, were very effective inhibitors and inhibited the formation of competent cells strongly when added at the beginning. These results indicate that de novo transcription and translation are required for competent cell formation as was suggested earlier (16). Competent cell formation and the process of transformation were not blocked by the drug added 60 min after transfer.

Tunicamycin (5 μg/ml), an antibiotic which inhibits the synthesis of cell wall materials (32, 35), lipid-P-P-*N*-acetylmuramic acid-pentapeptide, as well as teichoic acid (18), blocked competent cell formation. However, methicillin, a synthetic penicillin, an inhibitor of cross-linking in peptidoglycan (4), was not inhibitory at a concentration of 0.1 μg/ml, whereas the multiplication of noncompetent cells was blocked at this concentration. These results may suggest that the process of competent cell formation is dependent on the synthesis of wall materials but not on the final process of the cross-linking of peptidoglycan.

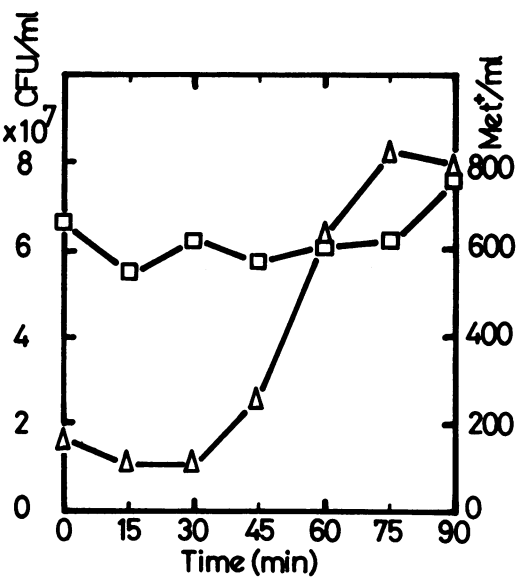

FIG. 2. Rate of development of competence. One milliliter of culture in SPII medium was incubated with transforming DNA (11.6 μg) for 5 min followed by DNase (250 μg) treatment for 3 min. Met$^+$ transformants were scored. CFUs were scored just before the incubation of the culture with DNA. Symbols: △, Met$^+$ transformant; □, CFUs.

TABLE 3. Inhibitory nature of antibiotics[a]

Drug (concn)	Time	Exp 1				Exp 2			
		Met^+/ml (10^2)	CFU/ml (10^6)	Frequency (10^{-4})	Ratio (0/60)	Met^+/ml (10^2)	CFU/ml (10^6)	Frequency (10^{-4})	Ratio (0/60)
HPUra	0	14	28	5.0	2.6	13	21	6.0	2.5
(10 μM)	60	11	54	1.9		14	59	2.4	
Rifampin	0	0.070	44	0.016	0.0053	1.0	95	0.11	0.017
(0.01 μg/ml)	60	19	61	3.0		39	88	4.4	
Chloramphenicol	0	0.090	50	0.018	0.023	0.50	52	0.096	0.044
(1 μg/ml)	60	5.5	69	0.80		11	73	1.5	
Tunicamycin	0	0.57	40	0.14	0.041	0.49	26	0.19	0.12
(5 μg/ml)	60	19	56	3.4		15	78	1.9	
Methicillin	0	3.5	22	1.6	2.6	2.8	16	1.7	1.8
(0.1 μg/ml)	60	3.5	57	0.62		14	84	1.6	
No drug		9.5	52	1.8		28	107	2.6	
No DNA		0.0050	71	0.00071		0.0050	148	0.00034	

[a] At the T_1 stage in SPI medium, NIG1121M cells were transferred to SPII medium. Drugs were added to the culture at 0 and 60 min. DNA was added at 60 min. After incubation for 30 min, DNA incorporation was terminated by the addition of DNase. The cells were collected by centrifugation at 10,000 × *g* for 5 min, washed once in SPII medium without drugs, and plated on selective agar to score Met^+ transformants. The number of CFUs was also scored for each sample.

The inhibitory nature of these drugs was not due to the release of DNase in the culture medium. A supernatant of a competent culture developed in the presence of inhibitor did not inactivate transforming DNA appreciably (data not shown).

Time of appearance of competent cells after transfer of early-stationary-phase cells into SPII medium. The accurate time of appearance of competent cells in SPII medium was measured with the inhibitors, rifampin, chloramphenicol, and tunicamycin. The drugs were added at 10- to 15-min intervals after the dilution of early-stationary-phase cells, and the eventual formation of competent cells was examined at 90 min by adding transforming DNA. Rifampin was used at a higher concentration (1.0 μg/ml) than that used for the experiment described in Table 3, because rifampin at a concentration of 0.01 μg/ml became ineffective during incubation for longer than 60 min.

These three drugs completely inhibited competent cell formation until about 50 min after transfer (Fig. 3). Around 50 min, competent cells began to appear at the time of the multiplication of viable cells. Rifampin was toxic toward noncompetent cells, and we could not accurately determine when noncompetent cells began to divide. On the other hand, tunicamycin blocked the multiplication of noncompetent cells without cell lysis and allowed us to determine when they began to divide. Thus, it is very probable that competent cell formation is in some manner related to cell division. Similar challenge experiments showed that HPUra (10 μM)-resistant cell multiplication began around 60 min after transfer, although HPUra did not block competent cell formation.

Development of competence in a D-alanine-requiring strain. D-Alanine is found in pentapeptides linked to *N*-acetylmuramic acid in cell walls (4), and D-alanine residues are required for cross-linking in the cell wall. We next examined the effect of D-alanine depletion in a D-alanine-requiring strain. Cells of the mutant strain were grown to T_1 phase in the presence of D-alanine and transferred to SPII medium with and without D-alanine. Competence developed even in the absence of D-alanine, whereas the total number of recipient cells decreased without D-alanine (Fig. 4a). To see whether or not competent cells of the D-alanine-requiring strain were formed with the synthesis of cell walls, tunicamycin was added to the culture, and its effect was examined. Tunicamycin completely inhibited the formation of competent cells until 50 min after transfer (Fig. 4b). This result indicates that the D-alanine-requiring strain needs cell wall synthesis to produce competent cells even in the absence of D-alanine in the medium. These results suggest that competent cells do not require the cross-linking of peptidoglycan in the synthesis of cell wall as suggested by the experiment with methicillin or that the formation of competent cells may be related to the presence or absence of cell wall accessory materials other than peptidoglycan because D-alanine is also found in some accessory components and its depletion may affect their synthesis. Tunicamycin also blocks their synthesis. Actually, teichoic acid (14) was found to act as a specific ligand for *B. subtilis* cell wall lytic enzymes,

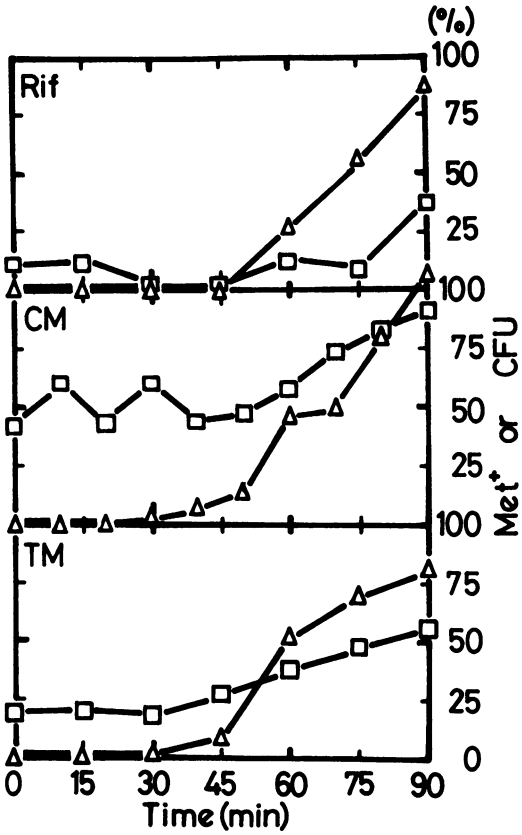

FIG. 3. Time of appearance of competent cells of NIG1121M in SPII medium. T_1-stage cells in SPI medium were transferred to SPII medium. Inhibitors were added at the time indicated. Competent cells were examined by addding transforming DNA at 90 min and incubated for 30 min followed by DNase treatment for 10 min. Cells were washed once with SPII medium free of drug as described in Table 3, footnote *a*, and Met^+ transformants and CFUs were scored. The number of transformants or CFUs was normalized by that of the sample which received no drug. Symbols: △, Met^+ transformants; □, CFUs; Rif, rifampin (1 μg/ml); CM, chloramphenicol (1 μg/ml); TM, tunicamycin (5 μg/ml).

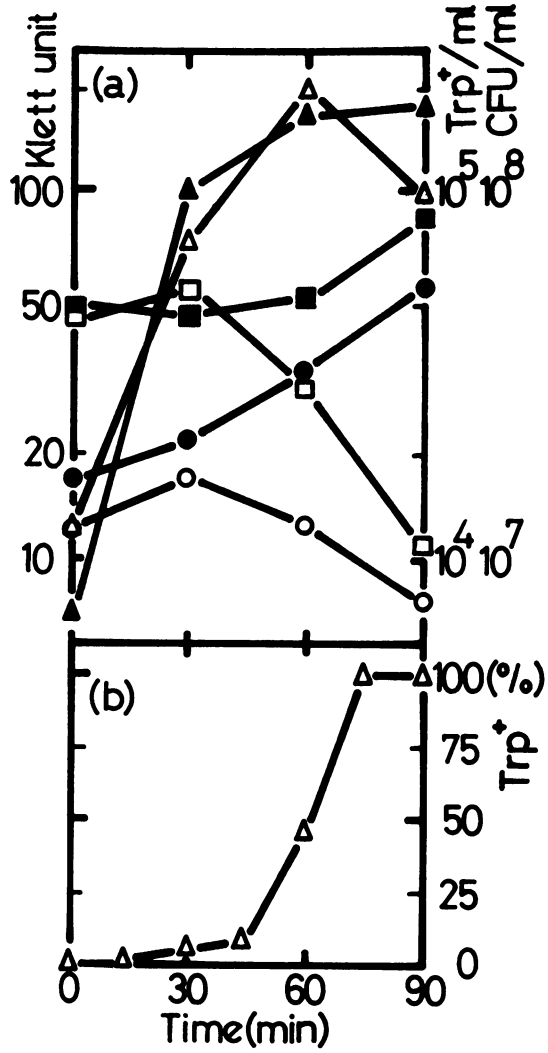

FIG. 4. Development of competence in D-alanine-requiring strain QB928 in SPII medium with or without D-alanine. (a) Cells of D-alanine-requiring strain QB928 were grown to T_1 stage in SPI medium supplemented with D-alanine and washed once in SPII medium without D-alanine. They were then suspended in SPII medium with or without D-alanine. Transforming DNA was added to 1 ml of competent culture. DNase was added after 30 min. Trp^+ transformants were scored on selective agar without Difco broth. Fresh SPII medium was used as a dilution buffer. Symbols: ○, turbidity without D-alanine; ●, turbidity with D-alanine; △, Trp^+ transformants without D-alanine; ▲, Trp^+ transformants with D-alanine; □, CFUs without D-alanine; ■, CFUs with D-alanine. (b) At the indicated time, tunicamycin (5 μg/ml) was added to the culture in SPII medium without D-alanine. The development of competence was measured as described in the legend to Fig. 3. The number of transformants was normalized to that of the sample which received tunicamycin at 90 min. Symbol: △, Trp^+ transformants without D-alanine.

which are assumed to be competence-inducing factors.

Blocking of competent cell formation at a sublethal concentration of ethanol, a specific inhibitor of early events of sporulation. *B. subtilis* cells form an asymmetric forespore septum after the end of log phase in a relatively poor medium which supports maximal sporulation (for a review, see references 15, 23). The addition of enough nutrient or the dilution of early sporulating cells with fresh medium might make sporulating cells resume vegetative cell division. The procedures adopted in the present experiment

TABLE 4. Activities of extracellular proteases[a]

Inhibitor	Activity (%)[b]	
	spo$^+$	*spo0A34*
+ 1,10-Phenanthroline (1 mM)	53.5	2.93
+ 1,10-Phenanthroline (1 mM) + Phenylmethylsulfonyl fluoride (2 mM)	3.22	1.04

[a] Protease activity was measured by the methods described by Dancer and Mandelstam (8). Strains NIG1121 (*spo0*$^+$) and NIG1131 (*spo0A34*) were grown to T_1 stage in SPI medium. Two milliliters of the culture supernatant were combined with 10 mg of hide powder (Sigma) in 1 ml of imidazol buffer and incubated at 37°C for 16 h with or without inhibitor. The sample was filtered, and the extinction of the filtrate was measured at 595 nm. Klett values of T_1-phase cultures of *spo0*$^+$ and *spo0A34* strains were 135 and 138, respectively.

[b] The activity was normalized with that of subtilisin (1 mg) (Sigma), which completely digested hide powder at 37°C for 20 min.

might make early sporulating cells resume new cell division. Because sporulation requires manganese (7, 34) at a relatively high concentration and sporulation is arrested before forespore septum formation in the absence of manganese (21), the effect of manganese on sporulation frequency and competence development was examined. The addition of manganese even at 1 μM increased the sporulation frequency of the cells in SPI medium from 10^{-6} to 10^{-2} (data not shown), whereas competence development was stimulated only to a small degree.

The activity of serine protease, which is thought to be a specific early-sporulation event (8) before forespore septum formation, was measured in the culture of the T_1 stage with which we examined the development of competence. The supernatant of the culture of the Spo$^+$ strain showed an activity of serine protease which was 1,10-phenanthroline resistant and was inhibited by phenylmethylsulfonyl fluoride (Table 4). 1,10-Phenanthroline is an inhibitor of metalloproteases, and serine protease is inhibited completely by phenylmethylsulfonyl fluoride. On the other hand, the *spo0* strain did not produce extracellular serine protease to an appreciable amount. Thus, it is very probable to consider early-stationary-phase cells in SPI medium as early sporulating cells, possibly before forespore septum formation.

Ethanol at 0.7 M reduces the growth rate of the cells without any effects on the final yield of cell density and blocks sporulation at stage 0, before forespore septum formation (5, 24). Forespore septum formation is specifically inhibited by ethanol, whereas normal cell division remains intact.

We examined the effect of ethanol on the formation of competent cells during growth in SPI and SPII medium. As shown in Fig. 5, 0.7 M ethanol completely inhibited the appearance of competent cells during vegetative growth in SPI medium (data not shown) and in SPII medium. The addition of 0.7 M ethanol to the competent culture grown in ethanol-free medium, just before the addition of transforming DNA, reduced the eventual number of transformants to 17% of that obtained without ethanol addition. Cells which received DNA were resistant to ethanol. Thus, the transformation of already competent cells seemed to be affeced by ethanol or competent cells seemed to be sensitive to ethanol. However, the complete absence of competence in an ethanol medium and the increasing appearance of competent cells in ethanol-free medium (Fig. 2) suggest that ethanol blocks the formation of competent cells. When early-stationary-phase cells grown in SPI medium in the presence

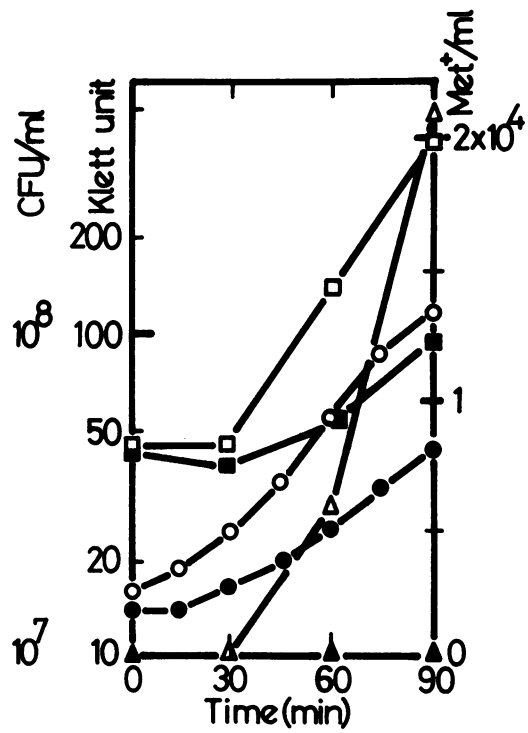

FIG. 5. Inhibitory nature of ethanol on competence development. Ethanol was added at a concentration of 0.7 M. NIG1121M cells were grown in SPI medium with ethanol to T_1 stage and transferred to SPII medium with or without ethanol. The development of competence was examined as described in the text. Symbols: ●, turbidity with ethanol; ○, turbidity without ethanol; ▲, Met$^+$ transformants with ethanol; △, Met$^+$ transformants without ethanol; ■, CFUs with ethanol; □, CFUs without ethanol.

TABLE 5. Development of competence of NIG1121M in medium which supports sporulation[a]

Medium[b]	Phase	Met^+ transformants per ml (10^3)	CFU/ml (10^7)	Frequency of transformation (10^{-4})	Probability[c]
Glucose NH_4Cl	Log	1.50	39.0	0.039	5×10^{-2}
	$T_{2.5}$[d]	1.83	4.40	0.42	
Glucose Casamino Acids	Log	1.70	27.1	0.063	3×10^{-5}
	$T_{2.5}$	1.60	8.60	0.19	
Glucose Histidine	Log	1.71	13.3	0.13	1.2
	$T_{2.5}$	63.0	16.5	3.8	
Glucose Glutamate	Log	60.5	15.1	4.01	2.5×10^{-2}–
	$T_{2.5}$	49.0	1.75	28.0	25×10^{-2}
Complex[e]	Log	<0.00500	13.4	0.0004	10^{-3}
	$T_{2.5}$	0.0500	27.0	0.0019	
Synthetic[f]	Log	21.5	30.0	0.72	
	$T_{2.5}$	90.0	13.3	6.8	

[a] An overnight culture in each medium was added as a 5% (vol/vol) inoculum into fresh medium. The cells were grown to T_1 phase and transferred to fresh medium. One milliliter of the culture was mixed with DNA to examine competence.

[b] Schaeffer's basal medium (30) was supplemented with carbon and nitrogen sources. Glucose, 0.2%; NH_4Cl, 0.05%; Casamino Acids, 0.1%; histidine, 0.1%; and glutamate, 0.1%.

[c] The probability for a cell to become committed to sporulation calculated by Schaeffer et al. (30).

[d] Cells of T_1 phase were diluted 10-fold with fresh medium and incubated for 90 min.

[e] A complex sporulation medium described by Schaeffer et al. (30).

[f] A synthetic medium for transformation described by Dubnau et al. (10).

of ethanol were transferred to ethanol-free SPII medium, competent cells developed, but their formation was reduced and retarded. The ethanol effect on the membrane is reversible (24). Thus, the process of competent cell formation is one of the early events during sporulation and requires an intact membrane. We also examined the activity of DNase which might be released in to the medium from cells grown in the presence of ethanol and could not detect any decrease of the activity of transforming DNA by incubation with the supernatant of the culture containing ethanol (data not shown). The development of competence in a D-alanine-requiring strain in the absence of D-alanine described in Fig. 4 was also inhibited by ethanol (data not shown). Thus, the formation of competent cells of the D-alanine-requiring strain is also inherent in the early sporulation stage.

Development of competence in medium which is favorable for sporulation. Generally, competent cells have been prepared in a specially devised medium which does not support optimal sporulation. On the other hand, sporulation occurs in a medium which does not support the development of competence. If competent cells are formed from early-phase sporulating cells by the supply of enough nutrients to resume new cell division, competent cells might be produced best in the sporulation medium by transferring the sporulating early-stage cells to fresh medium or by growing a vegetative culture with a high probability of sporulation. In 1965, Schaeffer et al. (30) proposed that the initiation of sporulation is under the control of catabolite repression. They examined the probability of sporulation in the continuous culture which contained a minimal synthetic medium with various kinds of carbon and nitrogen sources.

We examined the development of competence for transformation in such a medium. Cells were grown logarithmically in Schaeffer's basal medium (30) with various kinds of nitrogen and carbon sources, transforming DNA was added to the culture, and Met^+ transformants were scored. The wild-type strain showed fairly good competence for transformation in almost every synthetic medium (Table 5). In the medium with histidine as the sole nitrogen source, competence was comparable to that developed in SPI and SPII medium. In this medium, sporulation begins with an extremely high probability (30). Competence also appeared by diluting T_1-stage cells in fresh medium; this result again supports the idea that competent cells are formed from cells at an early sporulation stage. In a complex

TABLE 6. Frequency of transformation of *spo0* strains in synthetic medium

Genotype of recipient	Frequency of Met^+ transformation (10^{-4})	Frequency of[a] Km^r transformation (10^{-6})
Wild	8.9 (1.0)	1.1 (1.0)
spo0A	0.20 (0.022)	<0.24 (<0.23)
spo0B	0.047 (0.0053)	<0.023 (<0.012)
spo0C	3.9 (0.44)	0.36 (0.34)
spo0D	0.031 (0.0035)	
spo0E	0.19 (0.021)	1.6 (1.5)
spo0F	0.072 (0.0081)	0.0066 (0.0063)
spo0G	5.1 (0.58)	4.1 (3.9)
spo0H	0.12 (0.014)	0.14 (0.13)
spo0J	5.1 (0.57)	2.1 (2.0)
spo0K	0.015 (0.0017)	0.010 (0.0096)
Wild		1.7 (1.0)
spo0D		0.043 (0.025)
Wild		12 (1.0)
spo0A		0.18 (0.015)

[a] A competent culture (1 ml) was incubated with plasmid DNA (5.7 μg) at 37°C for 2 h to allow the expression of kanamycin resistance. The Km^r transformants were scored on minimal salts-glucose agar containing kanamycin (15 μg/ml) purchased from Meiji Co. Ltd (Tokyo, Japan). Plasmid DNA was purified from strain NIG1121 carrying pUB110 (27) as described by Gryczan et al. (13).

medium, the development of competence was very limited. Some factor(s) may function as a direct or indirect repressor for the expression of factor(s) required for the formation of competent cells.

Effect of *spo0* mutations on the development of competence. It is generally accepted that mutants defective in an early stage of sporulation show poor competence. However, their competence was not examined systematically under the same conditions (23). To see the effect of *spo0* mutations on the development of competence, we constructed 10 isogenic strains of different *spo0* loci (23) and examined the development of competence in SPII medium. The results shown in Table 6 revealed that most *spo0* mutations reduced the development of competence except for *spo0C*, *spo0G*, and *spo0J*. In *spo0B*, *spo0D*, *spo0K*, and *spo0F* strains, the development of competence was highly repressed. Thus, the development of competence depends on some *spo0* gene products during an early stage of sporulation. The poor competence of these *spo0* mutants is not due to the deficiency of recombinational ability. Competence for plasmid pUB110 (13)-mediated transformation from Km^s to Km^r was also poor for these strains, except for *spo0E* strain, which showed competence for plasmid DNA-mediated transformation. *spo0* strains used in the present study were resistant to UV light and chemical mutagens (data not shown). Because *rec* strains of *B. subtilis* were sensitive to the above agents (25), *spo0* mutants are considered to be proficient in recombinational ability.

DISCUSSION

With the method employed in the present study, competent cultures were induced from cultures returning to vegetative growth from an early sporulating (T_1) stage, possibly before forespore septum formation. T_1 stage cells are heterogenous in their ability to acquire the competent state. A small fraction of T_1 cells (which may have been committed to sporulation beyond some critical point) can only be induced for competence, whereas the majority of T_1 cells regain vegetative growth and are not induced in their ability to incorporate exogenous DNA. Therefore, the development of competence during vegetative growth is attributable to the small fraction of cells which have started to sporulate but fail to continue in the presence of sufficient nutrients in the medium.

The formation of competent cells from T_1-stage cells requires cell wall synthesis, which was inhibited by tunicamycin but not by methicillin or D-alanine depletion, whereas vegetative growth was blocked by these inhibitors. As the multiplication of viable cells and the formation of competent cells concomitantly became resistant to inhibitors after about 50 min in the SPII medium and some initiation mutants in cell division were defective in the development of competence (manuscript in preparation), it is very probable that cell wall materials required for competent cell formation are associated with cell division.

The sporulation of *B. subtilis* begins with a separation of the daughter chromosomes followed by the cessation of cell wall synthesis and the formation of an asymmetric forespore septum with less cross wall material between the two forespore membranes (15). Cell wall materials formed along the forespore membrane may be digested away by some autolysin specific to sporulation (15). That a mutant strain defective in more than 95% of total autolytic activity is still competent for transformation and proficient in sporulation (11) may suggest some sporulation-specific autolysins involved in competent cell formation and forespore septum formation. As suggested earlier, the site of forespore septum formation may be the site of the entry of transforming DNA (37), although we have no firm evidence in support of this hypothesis. The sporulation-specific nature of competent cell formation was also supported by the experiment with 0.7 M ethanol and some *spo0* mutations which blocked sporulation before forespore septum formation and the development of compe-

tence although we could not determine whether they block the development of T_1-stage cells in SPI medium or the formation of competent cells in SPII medium.

The formation of competent cells also requires de novo synthesis of RNA and protein. The development of competence may be accompanied by the activation of intracellular recombination ability involving the expression of a variety of genes responsible for DNA metabolism other than cell wall synthesis. Some inducible functions responsible for mutagenesis or prophage induction were found to operate in competent cells of *B. subtilis* (36).

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LITERATURE CITED

1. **Akrigg, A., S. R. Ayad, and J. Blamire.** 1969. Uptake of DNA by competent bacteria—a possible mechanism. J. Theoret. Biol. **24**:266–272.
2. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
3. **Archer, L. J., and O. E. Landman.** 1969. Development of competence in thymine-starved *Bacillus subtilis* with chromosome arrested at the terminus. J. Bacteriol. **97**:166–175.
4. **Blumberger, P. M., and J. L. Strominger.** 1974. Interaction of penicillin with bacterial cell: penicillin binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. **38**:291–335.
5. **Bohin, J. P., D. Rigomier, and P. Schaeffer.** 1976. Ethanol sensitivity of sporulation in *Bacillus subtilis*: a new tool in the analysis of the sporulation process. J. Bacteriol. **217**:934–940.
6. **Bott, K. F., and G. A. Wilson.** 1967. Development of competence in the *Bacillus subtilis* transformation system. J. Bacteriol. **94**:562–570.
7. **Charney, J., W. P. Fisher, and C. P. Hegarty.** 1951. Manganese as an essential element for sporulation in the genous *Bacillus*. J. Bacteriol. **62**:145–418.
8. **Dancer, B. N., and J. Mandelstam.** 1975. Criteria for categorizing early biochemical events occuring during sporulation of *Bacillus subtilis*. J. Bacteriol. **121**:411–415.
9. **Dedonder, R. A., J-A. Lepesant, J. Lepesant-Kejzlarova, A. Billault, M. Steinmetz, and F. Kunst.** 1977. Construction of a kit of reference strains for rapid genetic mapping in *Bacillus subtilis* 168. Appl. Environ. Microbiol. **33**:989–993.
10. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. **56**:209–221.
11. **Fein, J. E., and J. J. Rogers.** 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127**:1427–1442.
12. **Gass, K. B., R. L. Low, and N. R. Cozzarelli.** 1973. Inhibition of a DNA polymerase from *Bacillus subtilis* by hydroxyphenylazopyrimidines. Proc. Natl. Acad. Sci. U.S.A. **70**:103–107.
13. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
14. **Herbold, D. R., and L. Glaser.** 1975. Interaction of *N*-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. **250**:7231–7238.
15. **Hitchins, A. D.** 1978. Polarity and topology of DNA segregation and septation in cells and sporangia of the bacilli. Can. J. Microbiol. **24**:1103–1134.
16. **Kammen, H. D., R. J. Wojnar, and E. S. Canellakis.** 1966. Transformation in *Bacillus subtilis*. II. The development and maintenance of the competent state. Biochim. Biophys. Acta **123**:56–65.
17. **Mackenzie, J. M., M. M. Neville, G. E. Wright, and N. C. Brown.** 1973. Hydroxyphenylazopyrimidines: characterization of the active forms and their inhibitory action on a DNA polymerase from *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **70**:512–516.
18. **McArthur, H. A. I., F. M. Roberts, I. C. Hancock, and J. Baddiley.** 1978. Lipid intermediates in the biosynthesis of the linkage unit between teichoic acids and peptidoglycan. FEBS Lett. **86**:193–200.
19. **Nester, E. W.** 1964. Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis*. J. Bacteriol. **87**:867–875.
20. **Notani, N. K., and J. K. Setlow.** 1974. Mechanism of bacterial transformation and transfection. Prog. Nucleic Acid Res. and Mol. Biol. **14**:130–142.
21. **Oh, Y. K., and E. Freese.** 1976. Manganese requirement of phosphoglycerate phosphomutase and its consequences for growth and sporulation of *Bacillus subtilis*. J. Bacteriol. **127**:739–746.
22. **Okubo, S., and T. Yanagida.** 1968. Isolation of a suppressible mutant in *Bacillus subtilis*. J. Bacteriol. **95**:1187–1188.
23. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial sporulation. Bacteriol. Rev. **40**:908–962.
24. **Rigomier, D., J. P. Bohin, and B. Lubochinsky.** 1980. Effects of ethanol and methanol on lipid metabolism in *Bacillus subtilis*. J. Gen. Microbiol. **121**:139–149.
25. **Sadaie, Y., and T. Kada.** 1976. Recombination-deficient mutants of *Bacillus subtilis*. J. Bacteriol. **125**:489–500.
26. **Sadaie, Y., and K. Narui.** 1976. Repair deficiency, mutator activity, and thermal prophage inducibility in *dna-8132* strains of *Bacillus subtilis*. J. Bacteriol. **126**:1037–1041.
27. **Sadaie, Y., K. C. Burtis, and R. H. Doi.** 1980. Purification and characterization of a kanamycin nucleotidyltransferase from plasmid pUB110-carrying cells of *Bacillus subtilis*. J. Bacteriol. **141**:1178–1182.
28. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
29. **Schaeffer, P.** 1969. Sporulation and the production of antibiotics, exoenzymes and exotoxins. Bacteriol. Rev. **33**:48–71.
30. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. U.S.A. **54**:704–711.
31. **Spizizen, J., B. E. Reilly, and A. H. Evans.** 1966. Microbial transformation and transfection. Ann. Rev. Microbiol. **20**:371–400.
32. **Tamura, G., T. Sasaki, M. Mitsubashi, A. Takatsuki, and M. Yamasaki.** 1976. Tunicamycin inhibits the formation of lipid intermediates in cell-free peptidoglycan synthesis of bacteria. Agric. Biol. Chem. **40**:447–449.
33. **Tomaz, A.** 1969. Some aspects of competent state in genetic transformation. Ann. Rev. Genet. **3**:217–232.
34. **Vasantha, N., and E. Freese.** 1979. The role of manganese in growth and sporulation of *Bacillus subtilis*. J. Gen. Microbiol. **112**:329–336.
35. **Ward, J. B.** 1977. Tunicamycin inhibition of bacterial wall polymer synthesis. FEBS Lett. **78**:151–154.
36. **Yasbin, R. E.** 1977. DNA repair in *Bacillus subtilis*. II. Activation of the inducible system in competent bacteria. Mol. Gen. Genet. **153**:219–225.
37. **Young, F. E.** 1967. Competence in *Bacillus subtilis* transformation system. Nature (London) **213**:773–775.